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Gene delivery of human apolipoprotein E alters brain Aβ burden in a mouse model of Alzheimer's disease

Jean-Cosme Dodart*, Robert A. Marr†, Milla Koistinaho*, Beth M. Gregersen*, Seema Malkani*, Inder M. Verma†‡, and Steven M. Paul*‡

*Neuroscience Discovery Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285; and †Laboratory of Genetics, The Salk Institute, La Jolla, CA 92037

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Apolipoprotein E (apoE) alleles are important genetic risk factors for Alzheimer's disease (AD), with the ε4 allele increasing and the ε2 allele decreasing risk for developing AD. ApoE has been shown to influence brain amyloid-β peptide (Aβ) and amyloid burden, both in humans and in transgenic mice. Here we show that direct intracerebral administration of lentiviral vectors expressing the three common human apoE isoforms differentially alters hippocampal Aβ and amyloid burden in the PDAPP mouse model of AD. Expression of apoE4 in the absence of mouse apoE increases hippocampal $Aβ_{1-42}$ levels and amyloid burden. By contrast, expression of apoE2, even in the presence of mouse apoE, markedly reduces hippocampal Aβ burden. Our data demonstrate rapid apoE isoform-dependent effects on brain Aβ burden in a mouse model of AD. Gene delivery of apoE2 may prevent or reduce brain Aβ burden and the subsequent development of neuritic plaques.

amyloid plaques | gene therapy

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive function and is associated with a characteristic neuropathology, including amyloid plaques, neurofibrillary tangles, synaptic loss, and neurodegeneration. Mutations in several genes, including the presenilins 1 and 2 and the amyloid precursor protein (APP) gene, have been shown to cause rare autosomal dominant forms of AD (1–3). Moreover, mutations of these genes have been shown to alter normal processing of APP to the 4-kDa amyloid-β peptide(s) (Aβ), $Aβ_{1-40}$ or $Aβ_{1-42}$. AD mutations either increase production or alter the ratio of these peptides, which accumulate in the extracellular space to form amyloid-containing neuritic plaques. The apolipoprotein E (apoE) gene is a major risk factor for late-onset AD with the ε4 allele increasing and the ε2 allele decreasing the morbid risk for developing AD (4). Individuals carrying one or two ε4 alleles develop AD at a younger age and have higher amyloid-plaque burden compared with individuals carrying two ε3 alleles (5–8). In fact, several studies have demonstrated higher brain Aβ burden in elderly nondemented individuals carrying one or two ε4 alleles, suggesting that apoE4 somehow contributes to Aβ deposition and brain amyloid burden (9, 10). Genetic epidemiological studies also suggest a protective role for the ε2 allele, which in some studies has been shown to reduce the risk of AD by ≈50% (11).

ApoE is a 34-kDa lipid-binding protein produced primarily in the liver, which functions in the transport of triglycerides and cholesterol (12). ApoE is also abundantly expressed in the brain, primarily in astrocytes and microglia, where it has been postulated to play a role in neuronal plasticity and synaptogenesis (13–17). How apoE contributes to AD pathogenesis is, however, as yet unclear. We and others have shown that apoE facilitates Aβ fibrillogenesis and deposition *in vitro* and *in vivo* and/or participates in the clearance and degradation of Aβ in brain (18–23).

In the present study, we investigated whether gene delivery of the three common human apoE isoforms by using a lentiviral vector can directly alter the deposition of Aβ and the formation of amyloid plaques in PDAPP transgenic mice. We first demonstrate that intracerebral gene delivery of the lentivirus encoding (known as lenti- hereafter) apoE constructs results in efficient and sustained expression of human apoE in the hippocampus. We also demonstrate a significant isoform-dependent effect of human apoE on hippocampal Aβ burden and amyloid formation. PDAPP mice deficient in mouse apoE show increased Aβ burden after gene delivery of human apoE4. Moreover, in PDAPP mice expressing mouse apoE, direct intracerebral administration of lenti-apoE2 dramatically reduces hippocampal Aβ burden when compared with mice administered either the lenti-GFP or lenti-apoE4 constructs. Our data demonstrate a clear isoform-dependent effect of human apoE on brain Aβ pathology and suggest that gene delivery of human apoE2 may prevent and/or reduce brain Aβ burden and the subsequent formation of neuritic plaques.

Methods

Lentiviral Vector Production. Vector plasmids were constructed for the production of lentiviral vectors that express each of the human apoE isoforms (kindly provided by the laboratories of Sergio Fazio, Vanderbilt University, Nashville, TN, and Karl Weisgraber, University of California, San Francisco) and GFP (Fig. 7, which is published as supporting information on the PNAS web site). Lentiviral vectors were produced by using a four-plasmid transfection system as described in refs. 24 and 25. Briefly, 293T cells were transduced with vector and packaging plasmids, and the supernatants were collected and vectors concentrated by centrifugation. The lentiviral vector titers were estimated by measuring the amount of HIV p24 gag antigen with an ELISA kit (PerkinElmer) or by flow cytometry (for GFP vectors). Expression of apoE from vector-transduced cells was confirmed by immunoblots of cell culture supernatants by using an apoE specific antibody (E-19, Santa Cruz Biotechnology).

Transgenic Mice and Gene Delivery. All experiments were conducted in compliance with protocols approved by the Eli Lilly Institutional Animal Care and Use Committee. The transgenic mice used in this study were PDAPP mice overexpressing a human APP mutation (V717F) under the control of the platelet-derived growth factor promoter (26) and PDAPP mice lacking apoE (18). In a first series of experiments, lenti-vectors expressing GFP, apoE2, apoE3, or apoE4 were administered directly into the hippocampus of 8- to 9-month-old PDAPP mice expressing wild-type mouse apoE (n = 6–8) and 11- to 13-month-old PDAPP mice lacking apoE (n = 5–9). Mice were anesthetized with avertin and placed in a stereotaxic apparatus. Lentiviral preparations (4×10^9 units/ml) were injected bilaterally (2 μl per site) into the CA3 region of the hippocampus (−2.0 mm antero-posterior from bregma, ±2.3 mm medio-lateral from bregma, and 1.7 mm below dura). Mice were then individually housed and allowed to recover from surgery. Five weeks after

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Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; apoE, apolipoprotein E; APP, amyloid precursor protein.

‡To whom correspondence may be addressed. E-mail: spaul@lilly.com or verma@salk.edu.

injection, the mice were deeply anesthetized with avertin and transcardiacally perfused with heparinized saline. Brains were rapidly collected; one hemibrain was processed for histological analyses, and the other hemibrain was frozen on dry ice for biochemical analyses. In a second series of experiments, three groups of 10-month-old PDAPP mice were administered lentiviral preparations (GFP, apoE2, or apoE4) as described above. A fourth group of age-matched PDAPP mice, which did not receive any treatment, was used as an additional control. These mice (n = 7–12 per group) were killed 3 months after injection of lenti-vectors as described above. Finally, another cohort of PDAPP mice (7-month-old, n = 5 per group) were administered the lentiviral preparations into the CA1 region of the hippocampus (−2.0 mm antero-posterior from bregma, ±1.4 mm medio-lateral from bregma, and 1.3 mm below dura), and their brains were processed for analyses 5 weeks after treatment.

Tissue Preparation and Biochemical Analyses. To quantify Aβ, hippocampi were processed by using a three-step extraction procedure consisting of homogenizing samples in cold PBS, RIPA, and finally, 5 M guanidine-HCl as described in ref. 27. $A\beta_{1-40}$ and $A\beta_{1-42}$ were quantified in each pool by using a specific ELISA described in ref. 18. Proteins of interest were also analyzed by Western blotting. Briefly, proteins from RIPA extracts were size-fractionated by using 10% or 15% Tris·HCl SDS/PAGE (Criterion gel, Bio-Rad) and transferred onto poly(vinylidene difluoride) membranes. To detect apoE, the membrane was immunoblotted by using a biotinylated goat anti-human apoE antibody (0.02 μg/ml; BioDesign, Kennebunk, ME) followed by Neutravidin-HRP (1:100,000; Pierce) and reacted with west-femto SuperSignal (Pierce).

Tissue Preparation and Histological Analyses. Brains were drop-fixed in 4% paraformaldehyde for 4 h, transferred to 20% sucrose for 24–48 h, and frozen in liquid nitrogen. Saggital 20-μm-thick sections were cut at −18°C in a cryostat and placed on Superfrost slides. Brain sections were immunoreacted with one of the following antibodies: goat polyclonal anti-human apoE (Chemicon, 1:500), rabbit polyclonal anti-GFP (Chemicon, 1:500), rabbit polyclonal anti-glial fibrillary acidic protein (Chemicon, 1:1,000), mouse monoclonal 3D6 (recognizes free amino-terminal region of Aβ, 1:500), or rabbit polyclonal Aβ pan (BioSource International, Camarillo, CA; 1:250). The specificity of the immunoreactivity was confirmed by the lack of a signal when the primary antibody was omitted. The percentage of surface area covered by Aβ immunoreactivity was used to measure Aβ burden (27).

Alternate brain sections were rehydrated in PBS and treated with hematoxylin QS (Vector Laboratories) for 45 sec. After rinses in water, the sections were dipped in 95% ethanol and eosin (Richard-Allan Scientific, Kalamazoo, MI) for 10 sec. The sections were then rinsed in water, dehydrated by using an ascending series of ethanol and xylene, and coverslipped with permanent mounting media (Vectamount, Vector Laboratories). Congo red staining was performed as described by the manufacturer (Sigma Aldrich).

Results

Lentivirus-Mediated Expression of apoE in the Hippocampus. We first investigated whether both human apoE and GFP could be efficiently expressed in the hippocampus after intracerebral lentivirus-mediated gene delivery (see Fig. 7 for a diagram of the lentiviral vectors we constructed). PDAPP mice expressing mouse apoE or lacking apoE (apoE$^{-/-}$ mice) were administered the lenti-apoE or lenti-GFP vectors bilaterally into the hippocampus (either CA3 or CA1, see below) and subsequently killed for histological and biochemical analyses 5 weeks later.

In PDAPP mice lacking mouse apoE and administered the lenti-apoE vectors directly into CA3, immunostaining of brain sections by using an antibody directed against apoE revealed a diffuse pattern of immunoreactivity localized primarily to the hippocampus (Fig. 1 *A* and *B*). Although apoE immunoreactivity was more prominent at the injection site, intense immunolabeling was also observed over the entire hippocampus, particularly in the hilus of the dentate gyrus and along the mossy fibers projecting to CA3 (Fig. 1 *C–H*). Diffuse apoE immunoreactivity was also observed in the CA1 region, although much weaker than in the hilus of the dentate gyrus. The specificity of the immunoreactivity was confirmed by the lack of immunoreactivity in control brain sections (either omission of the primary antibody or immunostaining of brain sections from mice administered lenti-GFP vector). The diffuse patterns of apoE immunoreactivity suggest that apoE is mainly secreted from transduced cells; however, some staining in cell bodies within the hilus of the dentate gyrus was also detected. Hippocampal tissue from mice administered lenti-GFP (negative control) or lenti-apoE vectors were also analyzed by Western blotting along with hippocampal tissue from apoE targeted-replacement mice expressing more "physiological" levels of human apoE (28, 29). As shown in Fig. 2*A*, immunoblotting revealed a prominent 34-kDa band corresponding to apoE in virtually all mice administered the lenti-apoE vectors but not in PDAPP mice lacking

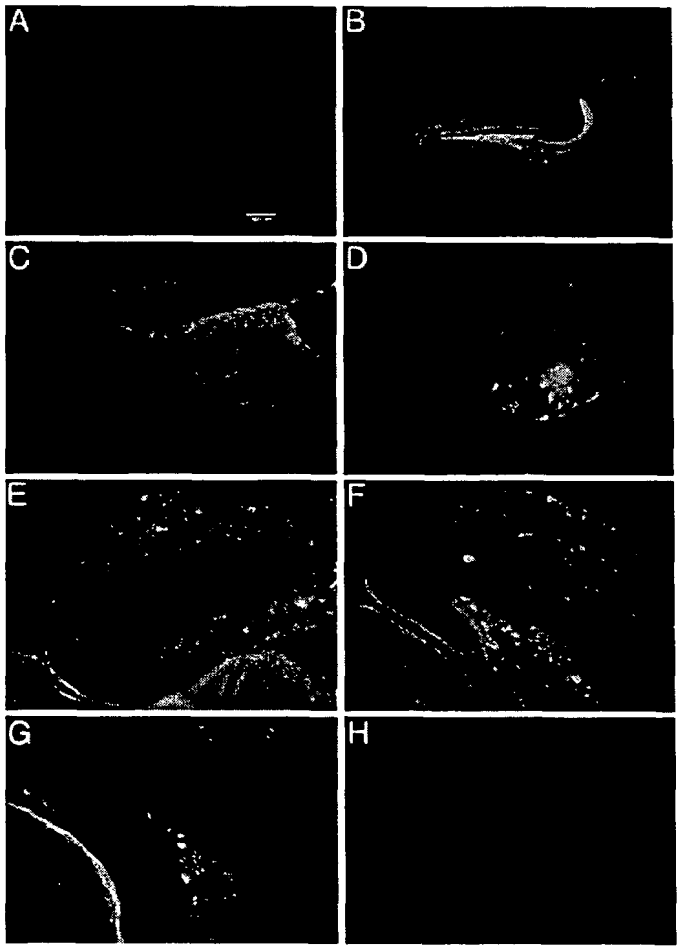

Fig. 1. Human apoE is expressed in the hippocampus of PDAPP mice lacking murine apoE after gene delivery. All mice were administered lentiviral vectors as described in *Methods* and studied 5 weeks later. (*A*) Brain section of a mouse administered lenti-GFP. Note the lack of apoE immunoreactivity. (*B*) Brain section of a mouse administered lenti-apoE4. Note the widespread area of apoE immunoreactivity (green) within the hippocampus. (*C–G*) Coronal brain sections of a mouse administered lenti-apoE2. Note that apoE immunoreactivity extends throughout the hippocampus from the most rostral region (*C* and *D*) to the most caudal region (*G*). Also note the lack of apoE immunoreactivity in the cerebellum (*H*).

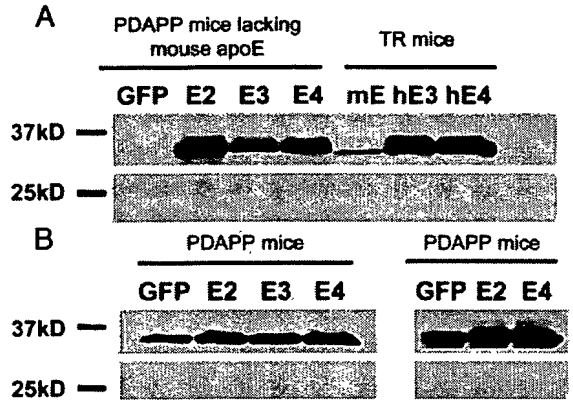

Fig. 2. Analysis of hippocampal apoE expression by Western blotting. Each lane was loaded with 32.4 or 20 μg total protein from RIPA-extracted hippocampal tissues for lentivirus-treated mice and targeted-replacement (TR) mice, respectively. (*A*) Hippocampal apoE expression 5 weeks after gene delivery in PDAPP mice lacking mouse apoE. Note the lack of signal in mice administered lenti-GFP (negative control) and the strong apoE immunoreactive band in mice administered lenti-apoE vectors. Immunoreactivity for apoE is comparable in lenti-apoE-treated mice and apoE TR mice expressing physiological levels of human apoE. (*B*) ApoE expression 5 weeks after gene delivery in PDAPP mice expressing mouse apoE by using 15% Tris·HCl (*Left*) or 10% Tris·HCl (*Right*) gels. Note the presence of an apoE immunoreactive band corresponding to mouse apoE in mice administered lenti-GFP. However, the signal for apoE appears to be increased in mice administered lenti-apoE vectors compared with mice administered lenti-GFP vectors. Moreover, the 10% Tris·HCl gels reveal an additional apoE immunoreactive band in mice administered lenti-apoE vectors.

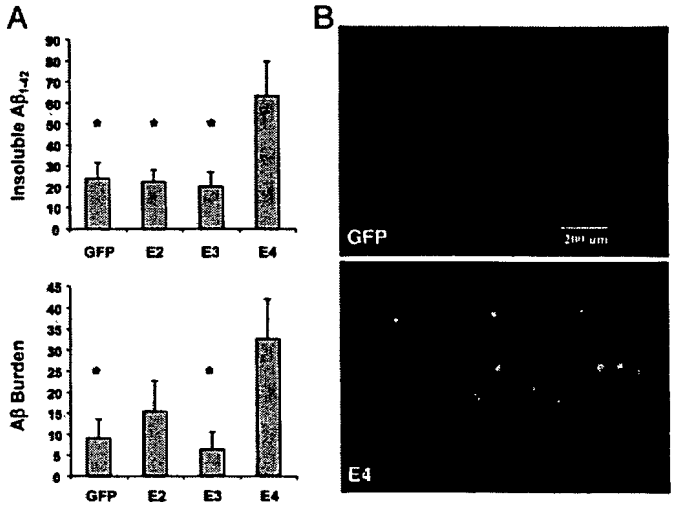

Fig. 3. Hippocampal $A\beta_{1-42}$ levels and Aβ burden are increased in lenti-apoE4-treated mice. (*A*) PDAPP mice lacking murine apoE show increased levels of hippocampal insoluble $A\beta_{1-42}$ and Aβ burden 5 weeks after apoE4 gene delivery (*, $P < 0.05$ versus E4; $n = 8$ for GFP, $n = 9$ for E2, $n = 8$ for E3, and $n = 5$ for E4). Hippocampal $A\beta_{1-42}$ levels were measured by a specific ELISA and Aβ burden by immunohistochemistry as described in *Methods*. Aβ levels are expressed in ng/mg protein, and values represent means plus SEM. Hippocampal Aβ burden represents the percent area covered by Aβ immunoreactivity. (*B*) Presence of Congo red-positive deposits in mice treated with lenti-apoE4. The percentage of mice showing congophilic amyloid deposits was significantly increased after lenti-apoE4 (*Lower*) treatment compared with lenti-GFP (*Upper*) treatment.

endogenous apoE and administered lenti-GFP (negative control). The amount of total protein loaded on the gel was slightly lower in samples from apoE targeted-replacement mice. Nonetheless, the amount of apoE in mice administered lenti-apoE vectors appeared very similar to those seen in the targeted-replacement mice (Fig. 2*A*).

In PDAPP mice expressing mouse apoE, analysis of human apoE expression after gene delivery was inconclusive because we observed strong cross-reactivities of our anti-apoE antibodies with both human and murine apoE. Indeed, no obvious qualitative difference in apoE expression could be observed between lenti-GFP- and lenti-apoE-treated PDAPP mice by immunohistochemistry (data not shown). However, the band for apoE detected by Western blotting appeared larger in mice administered the lenti-apoE vectors than in mice administered lenti-GFP (Fig. 2*B*). Moreover, when using 10% Tris·HCl gels, we also observed a band of slightly higher molecular mass corresponding to mature apoE in mice administered the lenti-apoE vectors (Fig. 2*B*) but not in mice administered lenti-GFP. This difference could be due to increased apoE expression, different posttranslational modifications of human apoE (30), or better reactivity of the anti-apoE antibody with human apoE than with murine apoE. Nonetheless, our data demonstrate that human apoE is efficiently expressed in lenti-apoE-treated PDAPP mice. Finally, double staining for GFP and the glial and neuronal markers, glial fibrillary acidic protein and neurofilament protein 70, respectively, revealed that neurons were the primary cell type transduced by the lentiviral vectors in our experiments (data not shown).

Gene Delivery of Human apoE4 Increases Brain Aβ Burden and Amyloid in PDAPP-apoE$^{-/-}$ Mice. We next examined whether expression of human apoE via the lenti-apoE vectors could alter brain Aβ burden in PDAPP mice lacking mouse apoE (Fig. 3*A*). In these mice, expression of lenti-apoE4 for 5 weeks resulted in a 2- to 3-fold increase in insoluble (guanidine extractable) $A\beta_{1-42}$ in the hippocampus ($P < 0.05$ versus GFP, apoE2, and apoE3) and a trend toward increased cell-associated (RIPA-extractable) $A\beta_{1-42}$ ($P < 0.06$ versus GFP and $P < 0.10$ versus apoE2 and apoE3). Lenti-apoE4 treatment also resulted in a significant increase in soluble (PBS-extractable) $A\beta_{1-42}$ ($P < 0.05$ versus GFP, apoE2, and apoE3). Moreover, $A\beta_{1-42}$ levels did not differ among groups administered lenti-GFP, lenti-apoE2, or lenti-apoE3. Hippocampal levels of $A\beta_{1-40}$ were also slightly increased in lenti-apoE4-treated mice but only in PBS-extractable ($P < 0.05$ versus lenti-apoE2 and lenti-apoE3 groups) fractions and RIPA-extractable ($P < 0.05$ versus lenti-apoE3) fractions (data not shown).

Finally, hippocampal Aβ burden (percent area covered by Aβ immunoreactivity) was also significantly increased in lenti-apoE4-treated mice compared with lenti-GFP- and lenti-apoE3-treated mice ($P < 0.05$, Fig. 3*A*). The presence of amyloid deposits (Congo red positive deposits) was observed in 80% of mice administered lenti-apoE4 compared with 0%, 33%, and 11% of mice administered lenti-GFP, lenti-apoE2, or lenti-apoE3, respectively (Kruskal-Wallis test: $P < 0.01$) (Fig. 3*B*). Only the lenti-apoE4-treated group was significantly different from the lenti-GFP-treated group (Mann–Whitney test: $P < 0.01$).

Human apoE2 Reduces Brain Aβ Burden in PDAPP Mice. We next asked whether gene delivery of human apoE into CA3 would alter hippocampal Aβ burden in PDAPP mice expressing endogenous mouse apoE. In these mice, expression of lenti-apoE2 for 5 weeks resulted in a 30–50% decrease in insoluble $A\beta_{1-42}$ and Aβ burden (Fig. 4). Analysis of insoluble Aβ by ELISA also revealed that mice treated with lenti-apoE2 had reduced levels of $A\beta_{1-42}$ compared with mice treated with lenti-apoE3 or lenti-apoE4 ($P < 0.05$) but to a lesser extent when compared with mice treated with lenti-GFP ($P = 0.12$, not significant). Insoluble $A\beta_{1-40}$ also showed a trend for a decrease in lenti-apoE2-treated mice; however, this did not reach statistical significance (data not shown). In these experiments, lenti-apoE4 treatment of PDAPP mice expressing mouse apoE did not increase hippocampal Aβ burden or insoluble $A\beta_{1-42}$. The

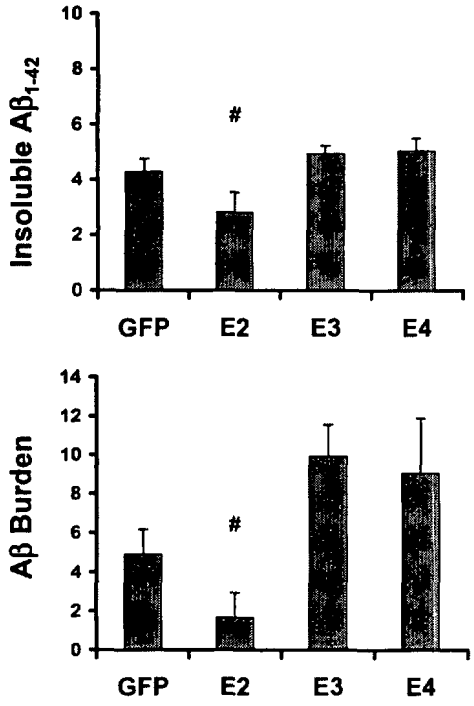

Fig. 4. Lenti-apoE2 treatment reduces hippocampal $A\beta_{1-42}$ levels (*Upper*) and Aβ burden (*Lower*) in PDAPP mice. PDAPP mice expressing murine apoE show decreased levels of insoluble $A\beta_{1-42}$ and Aβ burden 5 weeks after apoE2 gene delivery (#, $P < 0.05$ versus apoE3 and apoE4; $n = 6$ for GFP, $n = 6$ for E2, $n = 8$ for E3, and $n = 7$ for E4). Aβ levels are expressed in ng/mg protein, and values are expressed as means plus SEM. Hippocampal Aβ burden represents the percent area covered by Aβ immunoreactivity, and values are expressed as means plus SEM.

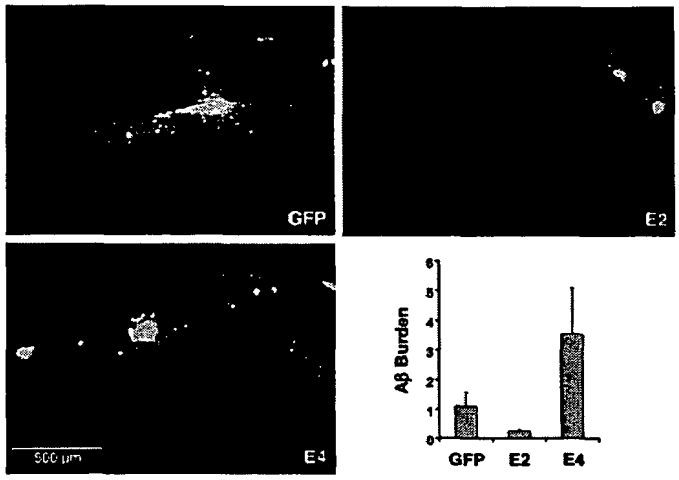

Fig. 5. Lenti-apoE2 reduces brain Aβ burden. Hippocampal Aβ burden in PDAPP mice after administration of lenti-GFP (*Upper Left*), lenti-apoE2 (*Upper Right*), or lenti-apoE4 (*Lower Left*) vectors into the CA1 region of the hippocampus. Brain sections were stained with an antibody (3D6) recognizing the amino-terminal region of Aβ (green) and counterstained with DAPI (nuclei, blue). (*Lower Right*) Hippocampal Aβ burden represents the percent area covered by Aβ immunoreactivity, and values represent means plus SEM. Note the rather marked difference between mice treated with lenti-apoE2 or lenti-apoE4 (unpaired *t* test, $P < 0.07$, $n = 5$ per group).

ELISA data paralleled the results obtained by quantitative immunohistochemistry (hippocampal Aβ burden). As shown in Fig. 4, lenti-apoE2-treated mice showed a significantly reduced Aβ burden compared with lenti-apoE3- or lenti-apoE4-treated mice ($P < 0.05$) and a nonsignificant decrease compared with lenti-GFP-treated mice ($P = 0.10$, not significant).

We next investigated whether treatment with lenti-apoE would alter hippocampal Aβ burden over a more extended period. Ten-month-old PDAPP mice were administered lenti-GFP, lenti-apoE2, or lenti-apoE4 into CA3 and killed 3 months later. A fourth group of age-matched PDAPP mice without surgery was analyzed in parallel as an additional control group. Because the Aβ burden and levels of insoluble $A\beta_{1-42}$ were comparable between the lenti-GFP-treated mice and age-matched PDAPP mice without surgery ($P = 0.33$ and $P = 0.80$, respectively; not significant), these two groups were combined and referred to as the "control group" for further analyses. Hippocampal Aβ burden was decreased by 61.5% and 50.2% in lenti-apoE2-treated mice compared with control mice ($P < 0.01$) and lenti-apoE4-treated mice ($P < 0.05$), respectively. Although insoluble $A\beta_{1-42}$ levels (ELISA) were decreased in the hippocampus of lenti-apoE2-treated mice compared with control mice (53.2% reduction) and lenti-apoE4-treated mice (47.3% reduction), this decrease did not quite reach statistical significance ($P < 0.08$, lenti-apoE2 versus control or lenti-apoE4).

To determine whether transduction of hippocampal neurons with lentiviral vectors resulted in histopathological changes, we stained alternate sections from our 3-month cohort with hematoxylin/eosin. To our surprise, we observed a loss of granule neurons of the dentate gyrus in most mice administered lenti-apoE vectors as well as lenti-GFP. Indeed, in this latter study (injection into the CA3 region of the hippocampus and analyzed 3 months later), we found a loss of granule neurons in the ventral and/or dorsal blades of the dentate gyrus (Fig. 8, which is published as supporting information on the PNAS web site). However, reexamination of brain sections from our first series of experiments (mice killed 5 weeks after lentivirus treatment) revealed very little, if any, to no loss of granule neurons (data not shown). The loss of granule neurons appeared to be due to the lentivirus preparation itself (and not the apoE) because no differences were observed between lenti-GFP and lenti-apoE treatments. Because such neurotoxicity could potentially confound the interpretation of our data demonstrating a rather dramatic difference between the lenti-apoE vectors on brain Aβ burden, we assessed whether alternative sites of lentiviral vector administration might mitigate or prevent granule cell loss. In a series of follow-up experiments, we observed little to no loss of granule neurons after administration of lenti-GFP into the CA1 region of the hippocampus of PDAPP mice (data not shown). Consequently, another cohort of 7-month-old PDAPP mice was administered lenti-GFP, lenti-apoE2, or lenti-apoE4 into the CA1 region and killed 5 weeks later (Fig. 5). As observed in our pilot experiments, by changing the site of injection, granule cell viability was preserved and we again observed good expression of lentiviral GFP throughout the hippocampus (data not shown). Consistent with our previous observations after administration into the CA3 region, PDAPP mice administered lenti-apoE2 into the CA1 region showed lower levels of hippocampal insoluble $A\beta_{1-42}$ and reduced hippocampal Aβ burden compared with lenti-apoE4 mice ($P < 0.06$ and $P < 0.07$, respectively). Despite the age difference between the mice used in our first study and in this study (9-month-old versus 7-month-old, respectively) and a difference between the injection sites (CA3 versus CA1, respectively), the two studies yielded very similar results. To compare results from both studies, we expressed the Aβ burden as a percent of control (i.e., the percentage hippocampal Aβ burden in lenti-apoE2- and lenti-apoE4-treated mice compared with lenti-GFP-treated mice) (Fig. 6). When combining the results of both studies, Aβ burden in lenti-apoE2-treated mice was markedly reduced compared with both lenti-GFP-treated mice ($P < 0.05$) and lenti-apoE4-treated mice ($P < 0.01$).

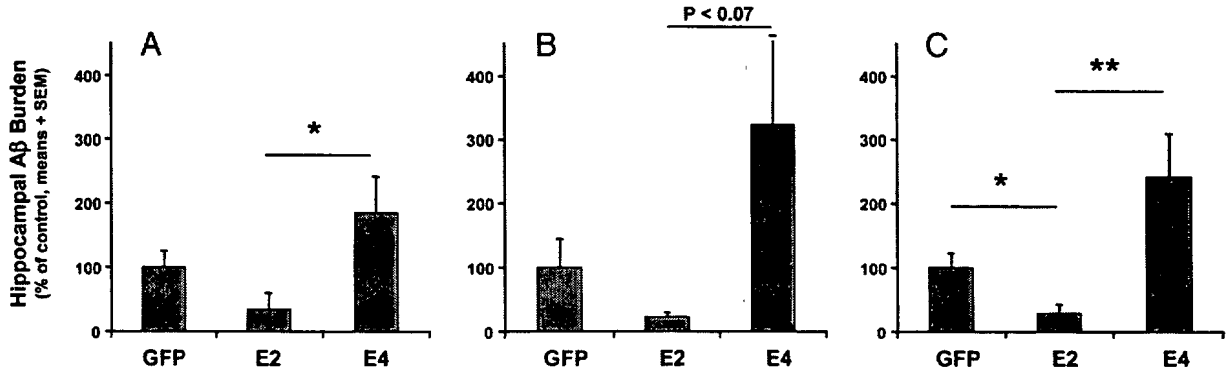

Fig. 6. Reduced hippocampal Aβ burden after lenti-apoE2 gene delivery. (*A*) PDAPP mice expressing mouse apoE were administered lentiviral vectors into CA3 (8- to 9-month-old mice; $n = 6$ for GFP, $n = 6$ for E2, and $n = 7$ for E4). (*B*) PDAPP mice expressing mouse apoE were administered lentiviral vectors into CA1 (7-month-old mice; $n = 5$ per group). (*C*) Overall reduction of hippocampal Aβ burden after combining results from both studies ($n = 11$ for GFP, $n = 11$ for E2, and $n = 12$ for E4). For each graph, Aβ burden is expressed as a percent of GFP control. *, $P < 0.05$; **, $P < 0.01$.

Discussion

In the present study, we investigated whether gene delivery of the three common human apoE isoforms directly into the hippocampus of PDAPP mice would alter Aβ and amyloid burden in an isoform-dependent manner. We first confirmed that lentiviral delivery of GFP and the three common apoE isoforms leads to efficient expression of these proteins in the hippocampus and, as reported in refs. 24, 31, and 32, that neurons are the primary cell type that is transduced by the lentiviral vector. The expression levels of apoE in the hippocampus were comparable with those of apoE targeted-replacement mice, which express "physiological" levels of apoE (28, 29). Interestingly, apoE immunoreactivity is not restricted to the injection sites (either CA3 or CA1) but rather is diffusely distributed and present throughout the entire hippocampus and is not associated with any specific cell type. By contrast, GFP immunoreactivity induced by lenti-GFP treatment was essentially localized to the injection site and expressed primarily in neurons. The diffuse and extended distribution of apoE immunoreactivity throughout the hippocampus suggests that neurons can synthesize and secrete the lentiviral-expressed apoE *in vivo*. In this regard, it is important to underscore that in rodents the vast majority of brain apoE is expressed in glia (astrocytes and microglia) and not in neurons (12, 33). However, it has also been reported that human brain apoE is expressed in certain populations of neurons as well (34, 35).

Direct intracerebral injection of lenti-apoE4 into the CA3 region of the hippocampus of PDAPP mice lacking apoE resulted in a significant increase in Aβ deposition by 2- to 3-fold and in a relatively short (5-week) period. Moreover, this increase in Aβ burden, measured by both immunohistochemistry and ELISA, was accompanied by an increase in amyloid (congophilic Aβ deposits) as well. These data suggest that, in the absence of mouse apoE, human apoE4 can promote Aβ deposition and fibrilization over a relatively short period. By contrast, the other apoE isoforms were less effective at promoting deposition or fibrilization over this time frame. These findings are consistent with a qualitatively unique "proamyloidogenic" effect of apoE4 compared with the other apoE isoforms, as has been suggested in refs. 19, 22, and 23 but has never been directly demonstrated *in vivo* until now. These data are obviously interesting in light of the important role the ε4 allele plays as a genetic risk factor for AD.

By contrast, lentiviral-mediated expression of human apoE2 for 5 weeks resulted in a rather robust reduction in hippocampal Aβ burden in PDAPP mice expressing mouse apoE. These data, suggesting a "dominant negative" effect of apoE2 over mouse apoE on brain Aβ burden, are interesting in light of our earlier findings in double transgenic mice that show a profound inhibitory effect of all three apoE human isoforms, but especially the apoE2 isoform, on Aβ deposition over an extended period (36). However, in the current study we did not observe a significant reduction in brain Aβ burden 5 weeks after treatment with lenti-apoE3 or lenti-apoE4. Differences in the ages of the PDAPP mice at the time of treatment with the lenti-apoE vectors, the presence or absence of mouse apoE, and the predominantly neuronal versus glial expression of apoE make direct comparisons of these findings difficult. Nonetheless, the rather robust effect of lenti-apoE2 treatment in reducing hippocampal Aβ burden in PDAPP mice is also noteworthy in light of clinical-epidemiological data suggesting that the ε2 allele is a protective genetic risk factor for AD. Thus, the protective effect of apoE2 on AD risk may in part be due to its ability to facilitate Aβ clearance and/or degradation in the brain and to prevent the formation of neuritic plaques, one of the neuropathological hallmarks of the disease. Moreover, the beneficial effects of apoE2 on brain Aβ burden are not simply due to the absence of proamyloidogenic apoE4 or apoE3 expression. It will be interesting to see whether other aspects of AD neuropathology (e.g., hyperphosphorylation of τ, neuritic plaques, etc.) are reduced after lenti-apoE2 treatment of PDAPP mice.

In our experiments, we observed a loss of granule neurons in the dentate gyrus after prolonged (3-month) treatment with the lentiviral vectors directly injected into CA3, and there was a rather profound loss of either (or both) ventral or dorsal blades of the dentate gyrus in some animals (Fig. 8). It should be emphasized, however, that the loss of granule neurons we observed was comparable when using each of the lentiviral constructs tested (including lenti-GFP). Moreover, PDAPP mice treated with lenti-GFP showed very similar brain Aβ levels compared with age-matched untreated PDAPP mice. Therefore, the marked differences in hippocampal Aβ and amyloid burden observed after lenti-apoE treatment were likely unrelated to differences in apoE expression and unlikely to be due to this loss of granule neurons *per se*. Moreover, we found that direct intracerebral injection of the lentiviral vector into the CA1 region did not result in loss of granule neurons, and the results on hippocampal Aβ burden were qualitatively and quantitatively very similar between the two injection sites. In related pilot experiments, we found that injection of lenti-GFP into CA3 did not result in a loss of granule neurons in Swiss-Webster mice (data not shown), suggesting a heightened vulnerability of granule neurons to the lentivirus in PDAPP mice. In this regard, several laboratories have recently reported hippocampal abnormalities and increased vulnerability of hippocampal neurons to a variety of insults in PDAPP mice (37–41). Furthermore, using the same type of lentiviral vectors as in the present study, Marr *et al.* (42) recently demonstrated good lentivirus-mediated expression of GFP, without toxicity, in another strain of APP transgenic mice.

Thus, the increased levels of Aβ itself in PDAPP mice may render granule neurons more vulnerable to lentiviral-mediated neurotoxicity. For the reasons stated above, we do not believe the neurotoxicity we observed after CA3 injection of the lentiviral vector (and which was highly variable from animal to animal) confounded the interpretation of our results, demonstrating clear apoE isoform-dependent effects on brain Aβ and amyloid burden.

Our findings also extend previous work from several laboratories, including our own, demonstrating apoE isoform-dependent effects on brain Aβ and amyloid burden *in vivo* and are consistent with human neuropathological findings in AD. The rather rapid proamyloidogenic effect of apoE4 expression observed in PDAPP mice lacking mouse apoE, however, was surprising in that it was observed as early as 5 weeks after treatment (the earliest time point studied) and was characterized by both nonfibrillar and fibrillar (congophilic amyloid) Aβ deposition. These findings were in marked contrast to the lack of effect of lentiviral-mediated expression of apoE2 and apoE3 under identical conditions and support a qualitative difference in the apoE4 isoform in the process of Aβ deposition and/or clearance and fibrilization. Finally, the rather rapid and unexpected reduction in hippocampal Aβ burden observed after treatment with lenti-apoE2 is interesting in view of the possible protective role of this apoE isoform. A lentiviral vector expressing neprilysin had been shown to reduce Aβ plaques when injected into the hippocampus of APP transgenic mice (42). Unlike neprilysin, a cell surface-associated endopeptidase, however, apoE is a secreted protein and therefore is likely to affect a much wider area of the CNS. Conceivably, the safe and effective expression of apoE2 in vulnerable brain regions in AD by using a similar viral (or other) vector could constitute a therapeutic approach to preventing or treating AD.

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